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13. ABSTRACT *(Maximum 200 Words)*

Tumor suppressors and oncogenes are central players in signal transduction pathways. Ras oncogene is one of the key intermediates that facilitates signal from upstream tyrosine kinases to a downstream cascade of serine/threonine kinases. The members of Rho family of Ras-related proteins are critical downstream components of signaling pathways. The Rho GTPases are involved in a wide spectrum of cellular functions. One of the regulators of Rho GTPases is Rho GTPase activating protein (GAP), which converts active Rho into inactive Rho. We have cloned a Rho GAP encoded by human chromosome 13q12. The sequence motifs of this Rho GAP were characterized and its expression profile in normal breast cell lines and breast carcinoma cell lines was determined. Its expression was much lower in carcinoma cell lines as compared to normal cell lines, and was undetectable in one of the carcinoma cell line tested. The Rho GAP message was highly sensitive to growth conditions. Thus mutations in Rho GAP, its reduced expression, and sensitivity to growth factors could likely enhance the levels of active Rho during breast tumorigenesis.

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Introduction:

The development of multicellular organisms is mediated by a highly complex and coordinated signaling network. The role of two sets of genes, namely, tumor suppressors and oncogenes has been described in the pathways that control normal development of a cell. This proposal addresses downstream elements of Ras signal transduction pathway that may mediate some aspects of malignant transformation. Ras- and Ras-related families, Rab, Rac and Rho are small GTP binding proteins. These proteins are key components of signal transduction pathways that link extracellular proliferation and/or differentiation signals such as growth factors and oncogenes to nuclear transcription of specific genes that promote these processes (1-3). The Rho pathway has been the object of much research into the mechanisms of this activation process both in normal and malignant cells. Indeed, mutations affecting Ras and/or Ras pathway are frequently seen in human cancers including lung, colon, endometrium, ovary, pancreas, thyroid and a smaller proportion of breast cancer (4). It has been demonstrated in several cancers where Ras is not mutated that downstream effectors of Ras signaling pathway are involved in tumorigenesis. Rho family of proteins is regulators of actin cytoskeletal organization. The activation of Rho by oncogenic Ras may affect cell-cell interactions and invasiveness that are characteristic of malignant cells (5,6). The Rho proteins constitute one of the major subdivisions of the Ras super family and comprise at least 14 known proteins (Rho A, B, C,D,E, 6,7, G, Rac 1, Rac 2, Rac 3, CDC42Hs, TC10 and TTF). These proteins bind and hydrolyze GTP and have low levels of intrinsic GTPase activity. Rho is required for growth factor induced formation of stress fibers and focal adhesions (7), and regulate cell morphology (8) cell aggregation (9) cell motility (10) and cytokinesis (11). The activity of Rho is negatively regulated by GTPase activating proteins (GAPs). We have cloned a Rho GAP from the BRCA2 region of chromosome 13q12 (12) and shown its aberrant expression in breast carcinoma cells. We have proposed Rho GAPs as having tumor suppressor activity and the current proposal is aimed at characterizing this Rho GAP and establishing its role in breast tumorigenesis.

Body:

The objectives of the funded project were to characterize a human Rho GTPase activating protein (GAP) that maps to chromosome 13q12 in the BRCA2 region. The technical objective that was stated to be partially accomplished during the first year of the grant is described below.

Technical objective 1:a) Investigate the expression profile and level of active Rho GAP expression in breast cancer cell lines and correlate lack of its activity to gene mutation, and b) Express the novel Rho GAP, and determine its biochemical and functional properties such as GAP activity, substrate

specificity, and phospholipase C- delta 1 stimulation. The progress on the various tasks is described against each task in the following section.

Task 1: Verification of the gene sequence and its further characterization.

A ~5Kb Rho GAP clone was isolated by screening a high quality lambda cDNA library. The insert from the lambda clone was isolated and introduced into a blue script plasmid for sequence determination. The plasmid clone was sequenced on both directions with T3 and T7 primers. Deletions were created in the clone by exonuclease digestion and the deleted clones were sequenced. The gaps in the sequence were filled by designing appropriate primers and extending the sequence further upstream or downstream. Gene fragment-overlapping programs were used to contig the various sequences and the full sequence was assembled. The nucleotide sequence is shown in **Figure 1**. The nucleotide sequence was translated to determine the protein sequence and the translated amino acid sequence is shown in **Figure 2**. The GAP domain in the sequence is highlighted. The full sequence was analyzed for the presence of various sequence motifs. The salient features in the sequence are described below. The protein shared significant homology with a rat Rho GAP that has phospholipase C-delta stimulation activity. The clone has several motifs that match with glycosylation sites, casein kinase and protein kinase C phosphorylation sites, tyrosine phosphorylation site, and interestingly several instability elements (ATTTA) in the 3' region of the message. The presence of these instability elements suggested that the stability of its message may be under the regulation of growth factors. Because Rho GAPs stimulate the intrinsic GTPase activity of Rho proteins, loss of Rho GAP activity can potentially allow for the Rho proteins to remain in activated state and thereby enhance the activity of downstream players responsible for cell growth and proliferation.

Task 2: Rho GAP expression profiles in various breast carcinoma cell lines.

A number of breast cancer cell lines were evaluated for the expression of Rho GAP message. In order to ascertain the full message in these cell lines RT-PCR was conducted with the primers specific to the 5' region of the cDNA. The poly A+ RNA was isolated by using an oligo dT matrix and the amount of RNA was determined by absorbance at 259 nm wavelength. Comparable amounts of RNA were reverse transcribed by using oligo dT as a primer, and the cDNA was then amplified with primer pairs specific to the 5' sequence. Control amplifications were carried out with the same template by using 5' primers specific to transferrin receptor gene, which also is a 5 Kb transcript. The breast carcinoma cell lines namely, T-47D, MDA-MB 231, MDA-MB-361, and MDA-MB-463 were

used for these studies. As shown in **Figure 3**, the Rho GAP transcript was not detectable in MDA-MB-231 under the PCR conditions employed, and the levels of gene expression were variable in the other cell lines. The abundance of Rho GAP message in breast carcinoma cell lines were relatively lower than the cell line MCF-10A established from normal breast epithelial cells. These results indicate that Rho GAP levels respond to the normal or tumor status of breast cells. Further evaluation of quantitative expression of Rho GAP transcript in a variety of cell lines will allow generalization of Rho GAP expression in breast carcinoma.

Based on the presence of instability elements in the Rho GAP sequence, we reasoned that the message stability may be affected by the composition of the growth medium. We, therefore, allowed the cell line MDA-MB-463 to grow in the presence of 10%, 8%, 6% and 5% serum, respectively. The cells were harvested and processed for RNA isolation. Equal amounts of RNA was subjected to RT-PCR with primers specific to Rho GAP for either 30 cycles or 35 cycles. As shown in **Figure 4**, the message was marginally detectable after 35 cycles of amplification in cells grown in the presence of 5% serum. The amplification of GAPDH, a housekeeping gene, was not affected under these growth conditions. These results indicate that the stability of Rho GAP is affected by the composition of the growth medium.

Task 3: Correlation of GAP activity by nitrocellulose assay with SSCP gene mutation.

The results of transcript profiling above suggested that the transcript abundance is one of the indicators of the functional product. However, it's necessary to ascertain the sequence of the transcript in various cell lines. The absence of the transcript as shown in one of the cell lines, clearly indicates that the gene is not expressed in detectable levels in this cell line. We, therefore, performed a mutational analysis on exonic sequences of the various transcripts. The primers used to amplify various exons are described in **Table 1**. The various exons were amplified in the presence of radiolabeled dCTP. The amplification was carried out in a standard PCR mixture containing 1X PCR buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3) and 2.5 mM magnesium chloride, 0.2 mM dNTPs, 20 microCi of alpha-32P labeled dCTP, 0.2 uM each of the flanking primers, 1 unit of Taq DNA polymerase, and 0.5 fmol of preamplified template. The reaction mixture was heated at 94^0 C for 2 min and then subjected to 20 cycles of 94^0 C/45 sec and 65^0 C or 60^0 C/1 min. An aliquot of 5 ul of the amplified product was treated with 1 unit of Klenow polymerase for 10 min at 37^0 C to remove 3' extraneous nucleotides and to degrade unused nucleotides. An aliquot of the amplified mixture was denatured and applied to a sequencing gel and electrophoresed at 40 W in 0.5X TBE-5% glycerol. The gel was removed and

transferred to a filter paper, dried and exposed for autoradiography. An aberrant mobility in exon 8 was observed in MDA-MB-231 cell line (**Figure 5**). We have currently undertaken complete sequencing of Rho GAP from this cell line and two more cell lines where the SSCP analysis yielded ambiguous results.

We have been analyzing the GTPase activity profile of these cell lines by using a nitrocellulose overlay assay. The protocol for this assay is shown in **Figure 6.** Briefly, proteins in the cell extract are resolved by SDS-PAGE and electroblotted on to a nitrocellulose filter. A fraction of the GAPs refold after binding to the nitrocellulose filter. The filter is soaked in the assay buffer solution containing [gamma- ^{32}P] GTP- loaded Rho protein. The Rho binds to its cognate RhoGAP and leads to hydrolysis of GTP that is bound to Rho. Hydrolysis of bound GTP around the bands corresponding to GAPs leaves label-free areas as shadows on the nitrocellulose filter. The filter is placed on 1% agar plate to absorb the excess liquid. A replica nitrocellulose filter is then made from the original filter and exposed for autoradiography. GAPs are visualized as a white band against a dark background, and compared to standard lane. Although our initial experiments have revealed inconclusive results, as multiple bands were observed in the expected size range, a qualitative difference in the GAP activity is detectable. We are currently modifying this protocol to suit the experimental conditions and including the recombinant Rho GAP for comparison.

Task 4: Purification of proteins Rho GAP, Rho A and phospholipase C-delta.

The coding sequences for Rho GAP, Rho A and phospholipase C-delta were PCR amplified from the cDNAs, and the ends of these amplicons were repaired . The entire coding region was subsequently cloned in the bacterial expression vector pGEX (Pharmacia) for expression and purification of Rho GAP, RHO A, and phospholipase C-delta proteins. The recombinant clones were tested by restriction digestion and confirmed to have insert sizes of ~3.0 Kb, ~ 1.8 Kb and ~2.3 Kb, respectively. These inserts represent the expected sizes. In addition to the insert, a 4.9 Kb vector band was clearly separated in the digests. The authenticity of the inserts was further confirmed by partial sequence determination.

FIGURE 1

Nucleotide Sequence of RhoGAP

TGACTGGCTCCGTGCTGCCGGGTTCCCGCAATACGCTCAGTTATATGAGGATTCACAATTTCCCATCAACATTGTGGCTGTCAAGAATGATCATGATT
TTCTTGAAAAGGACCTTGTAGAACCTCTTTGCAGACGACTAAATACGTTGAACAAGTGTGCCTCA**ATG**AAACTTGATGTGAACTTCCAAAGGAAAAAG
GGTGACGACTCCGATGAGGAAGATCTTTGTATCAGCAACAAATGGACTTTCCAAAGAACCAGTCGCAGGTGGTCTCGTGTGGACGACCTCTACACGCT
GCTCCCTCGAGGAGACAGAAATGGGTCACCGGGAGGCACGGGGATGAGGAACACGACCAGCAGTGAGAGCGTCCTCACAGACCTGAGCGAGCCTGAGG
TCTGCTCCATTCACAGCGAAAGCAGTGGAGGCAGCGACAGTCGCAGCCAGCCGGGCCAGTGCTGTACAGACAACCCGGTCATGCTGGATGCCCCACTC
GTCAGCAGCAGCCTCCCACAGCCCCCCAGAGATGTCCTCAACCACCCCTTCCACCCCAAGAATGAGAAGCCCACGAGGGCTAGGGCCAAATCATTTTT
GAAACGCATGGAAACACTCCGAGGGAAGGGAGCCCACGGGAGGCATAAGGGGTCTGGGCGGACAGGTGGCCTGGTGATCAGTGGGCCCATGTTGCAGC
AGGAGCCAGAGTCCTTTAAGGCTATGCAGTGCATCCAAATACCAAATGGAGATCTCCAGAATTCGCCGCCACCTGCCTGCAGAAAAGGGCTCCCATGC
TCTGGCAAGTCGAGTGGCGAGAGCAGCCCGTCGGAGCACAGCAGCAGCGGGGTGAGCACGCCCTGCCTGAAGGAACGCAAGTGCCACGAGGCCAACAA
GCGCGGGGGCATGTACTTGGAGGACCTAGATGTGCTGGCGGGGACAGCACTGCCGGATGCAGGGGACCAAAGCCGTATGCATGAGTTTCACTCCCAAG
AGAATTTGGTGGTGCATATTCCCAAGGATCACAAACCAGGAACATTCCCCAAGGCACTTTCTATTGAAAGCCTCTCTCCCACAGATAGTAGCAATGGG
GTTAATTGGAGGACCGGTAGCATCTCCCTGGGCAGAGAGCAGGTCCCTGGTGCCAGGGAGCCCCGGCTCATGGCGTCCTGCCACAGAGCCAGCCGAGT
CAGTATCTATGACAATGTCCCTGGCTCCCATCTGTATGCCAGCACAGGAGATCTTTTGGACTTGGAGAAAGATGACCTTTTCCCTCACTTGGATGACA
TTCTGCAGCATGTCAATGGGCTCCAAGAGGTAGTCGATGACTGGTCCAAAGATGTCTTGCCTGAACTGCAAACTCATGATACATTGGTTGGGGAACCT
GGCTTATCCACCTTTCCATCTCCTAATCAGATCACCTTAGATTTTGAAGGTAACTCTGTCTCAGAAGGTCGGACGACACCCAGTGATGTGGAAAGAGA
TGTAACATCTCTTAATGAATCTGAGCCTCCTGGGGTCAGAGACAGGAGGGATTCTGGTGTAGGGGCCTCTCTGACCAGGCCAAACAGGCGACTCCGAT
GGAACAGTTTCCAGCTGTCGCACCAGCCCCGGCCGGCCCCAGCATCGCCCCACATCAGCAGCCAGACGGCCAGCCAGCTGAGCCTGCTCCAGCGCTTC
TCACTGCTCCGCCTCACGGCCATCATGGAGAAGCACTCCATGTCCAACAAGCACGGCTGGACATGGTCAGTTCCAAAGTTCATGAAGAGGATGAAAGT
TCCCGACTACAAAGACAAGGCTGTCTTTGGCGTTCCTCTCATAGTCCACGTCCAAAGAACGGGACAGCCCCTGCCTCAAAGTATTCAGCAAGCACTGA
GATATCTACGCAGCAACTGCCTCGATCAGGTGGGTCTTTTTCGCAAATCAGGAGTGAAGTCTCGAATCCATGCCCTTCGCCAAATGAATGAAAACTTC
CCTGAGAACGTCAACTATGAAGACCAGTCTGCTTATGATGTGGCGGATATGGTGAAACAGTTCTTCCGGGACCTCCCTGAGCCTCTTTTCACCAACAA
GCTCAGTGAGACCTTTCTCCATATCTATCAGTATGTCTCCAAAGAGCAGCGGCTGCAGGCCGTGCAGGCTGCCATCCTGCTACTGGCCGATGAGAACA
GGGAGGTCCTGCAGACGCTCTTGTGTTTCCTGAACGACGTCGTCAACTTGGTGGAAGAGAATCAGATGACGCCCATGAACCTGGCAGTGTGTCTGGCC
CCCTCCCTCTTTCATCTTA**ATTTA**TTGAAGAAAGAAAGCTCTCCACGAGTCATACAGAAGAAATATGCCACTGGGAAGCCAGATCAAAAGGACCTCA
ACGAGAATCTGGCAGCAGCTCAGGGGCTAGCGCACATGATCATGGAATGCGACAGACTTTTTGAGGTTCCACACGAGTTGGTGGCCCAGTCTCGTAAC
TCGTATGTGGAGGCTGAGATCCACGTGCCAACCCTGGAAGAATTGGGGACACAGCTGGAGGAGAGTGGGGCAACTTTCCACACTTACCTGAACCATCT
CATCCAGGGCCTCCAGAAAGAAGCCAAGGAGAAGTTCAAAGGATGGGTCACGTGCTCCAGCACGGACAATACAGATCTTGCTTTCAAAAAGGTGGGCG
ACGGGAACCCGCTGAAGCTGTGGAAGGCTTCTGTGGAGGTGGAAGCACCCCCCTCAGTGGTCCTGAACCGCGTGCTGAGAGAGCGCCACCTGTGGGAC
GAGGACTTTGTGCAGTGGAAGGTTGTGGAAACTCTAGACAGGCAAACAGAGATCTACCAGTATGTGCTGAACAGCATGGCTCCCCATCCTTCCAGAGA
CTTTGTGGTTCTCAGGACCTGGAAAACTGATTTGCCCAAAGGAATGTGTACCCTGGTGTCCCTCTCCGTGGAGCATGAGGAAGCCCAGCTCCTGGGTG
GTGTGCGAGCAGTGGTGATGGACTCGCAGTACTTGATAGAACCGTGTGGCTCTGGCAAGTCAAGACTGACTCACATCTGCAGGATAGACCTGAAAGGT
CACTCCCCAGAATGGTACAGCAAAGGCTTTGGACATCTGTGTGCAGCAGAAGTTGCCAGGATTAGAAACTCTTTCCAGCCCCTCATTGCTGAGGGCCC
AGAAACTAAAATC**TGA**GTTTTGCCCAGTGTGACATCAAACTCAGGGAAGAGGAAGCTAAAGTGACGAGTGTGGCAGAGAGTGTGCATGTGAGAAAGCG
AGAGAAAGAGGAACTGAAGGACGCGGTTAATGCCTAAAAATGGAAACGTTAAGAAGTTGGAATGTTGGAGATGCAAGAATTTCCAAGAACTTTCTTAG
CCTTCCTGGAGATGGCTACATCCCTACTAATATAATTTTAAAATGAGAACTTTATATATATTACTTAAAATTAAATGGACTATTCCTTGTGCATTGCC
TAATTTGCT**ATTTA**AAGGCTTCTAAGAAGCGTATACCTAACTGTA**ATTTA**TGTATGTATAGCATATGTACATATGTGTGTATATCTCCATCTTTACT
GTATATATGTAAAATACCAATTTTATATAGAATTGTGTGTTTTGAAAATGACGGTGTCTGACTCAGTGAGTCCCTTCCTCACACAGTTCTTTCCAAGT
GGCTCTGGGCCCCATCTCTCCACTGTCCTGTAAGCTGTGCAGAACCTGCTGCTAACACCAAGGTGTGAACATGCCCTGATGCCTAACCAAAGATGAGT
TAACCAAAGGAAAATAACATTAAAGGAGACTTATGTGTTAACGCTTTGTTTCTGCTATTCAAAAACTGAGAGTGGAGATCTGGGATAAAGCAAGGAAA
TAATAATTACTCCTCCTTAAAGCAAATGGGGGGGTGAGAAGTCATTACCAA**ATTTA**AAGCTAGATGAGGAGTTGCCACTGGGCCCAGTAAGATGGAA
TTTCAGTGAGATATGGACCACCGGAGTCAGCGAGAGTGACTGAAACAGAAGCGATACCTCTCGCTCCCATGCCCATCACTACAGACCCCAAGTCAAGA
TGAATATCATAGCCTTTACTTCTTCACAGCCAAAGGGAGCCCCTGTGTTGTCTCAAGTTTTTATAAATACATTTCATAATGTTATTAAATGTCATTCT
ATTTGACCAGTGGCCTATTTGGTCACAGTTAATTGGTGTTTTCTTATTGCACTGAATTCAACTCCAGACACCATACAAAGGGAGATGATGGCCATTCC
GTTCAAATCCTAGATCGTTACAGCTTCAGGGAATTCATATTTTGTTATGTGTAGGATACTCTTAAAATGTAATTCATTAAACTTTTACAATCTGAAAG
ACAGGGTTTTTAACTAACATGAGACCAAAACTATGTTCTTTGATTAGTTTTAGATAGTATAATCGGGTTTATTAATTCTTCTGTGTTTCTTCACTAGC
CAGTCCAAGCTACCTATGCATTTGACCCAACCTT**ATTTA**TTATTGTACAGATGAAGCGAATTGACTCCCTTTAGCCAACTGCTAATGGATCGAATGT
GCTTTTTATTGTAATTCAACAGCTATAGAGAGAAAGATAACTTATTGTGTGTTTGATTTCAGGGAGAGAGATTTTCTTTGGTCATCCATAATAGAGAT
TGATAAG**ATTTA**GCAACTGGTGTTGGAGAAAAAAAGAAAAGCAAA

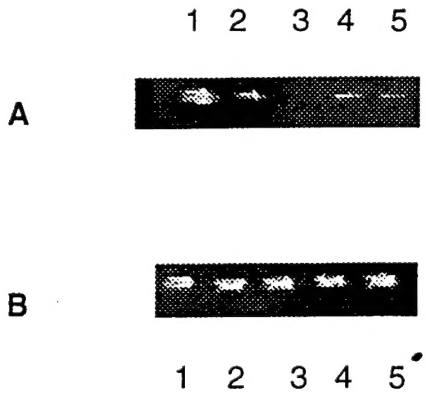
FIGURE 2

Predicted Amino Acid Sequence of Rho GAP

- MKLDVNFQRK KGDDSDEEDL CISNKWTFQR TSRRWSRVDD LYTLLPRGDR NGSPGGTGMR NTTSSESVLT DLSEPEVCSI HSESSGGSDS RSQPGQCCTD NPVMLDAPLV SSSLPQPPRD VLNHPFHPKN EKPTRARAKS FLKRMETLRG KGAHGRHKGS GRTGGLVISG PMLQQEPESF KAMQCIQIPN GDLQNSPPPA CRKGLPCSGK SSGESSPSEH SSSGVSTPCL KERKCHEANK RGGMYLEDLDVLAGTALPDA GDQSRMHEFH SQENLVVHIP KDHKPGTFPK ALSIESLSPTDSSNGVNWRT GSISLGREQV PGAREPRLMA SCHRASRVSI YDNVPGSHLY ASTGDLLDLE KDDLFPHLDD ILQHVNGLQE VVDDWSKDVL PELQTHDTLV GEPGLSTFPS PNQITLDFEG NSVSEGRTTP SDVERDVTSL NESEPPGVRD RRDSGVGASL TRPNRRLRWN SFQLSHQPRP APASPHISSQ TASQLSLLQR FSLLRLTAIM EKHSMSNKHG WTWSVPKFMK RMKVPDYKDK AVFGVPLIVH VQRTGQPL**PQ SIQQALRYLR SNCLDQVGLF RKSGVKSRIH ALRQMNENFP ENVNYEDQSA YDVADMVKQF FRDLPEPLFT NKLSETFLHI YQYVSKEQRL QAVQAAILLL ADENREVLQT LLCFLNDVVN LVEENQMTPM NLAVCLAPSL FH**LNLLKKES SPRVIQKKYA TGKPDQKDLN ENLAAAQGLA HMIMECDRLF EVPHELVAQS RNSYVEAEIH VPTLEELGTQ LEESGATFHT YLNHLIQGLQ KEAKEKFKGW VTCSSTDNTD LAFKKVGDGN PLKLWKASVE VEAPPSVVLN RVLRERHLWD EDFVQWKVVE TLDRQTEIYQ YVLNSMAPHP SRDFVVLRTW KTDLPKGMCT LVSLSVEHEE AQLLGGVRAV VMDSQYLIEP CGSGKSRLTH ICRIDLKGHS PEWYSKGFGH LCAAEVARIR NSFQPLIAEG PETKI*

FIGURE 3

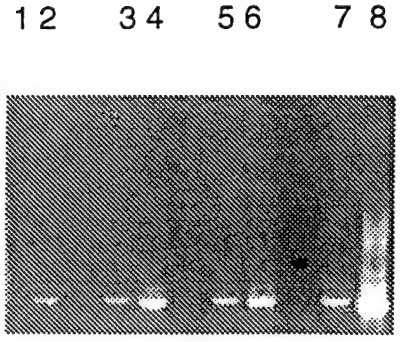
Rho GAP EXpression in Various Breast Cell Lines



RNA was isolated from cell lines MCF-10A (lane 1), T-47D (lane 2), MDA-MB-231 (lane 3), MDA-MB-361 (lane 4), and MDA-MB-468 (lane 5) and subjected to RT-PCR with Rho GAP 5' specific primers for 35 cycles (panel A), and with transferrin receptor 5' specific primers for 35 cycles (panel B). An aliquot of the amplified mixture was electrophoresed in an agarose gel. Equal amounts of RNA was used.

FIGURE 4

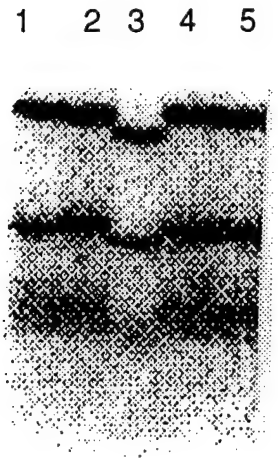
Growth Medium Dependance of Rho GAP Expression



The cell line MDA-MB-463 was grown in the presence of 5% (lanes 1, 2), 6% (lanes 3, 4), 8% (lanes 5, 6), and 10% (lanes 7, 8) serum. The cells were harvested and processed for RNA isolation. Approximately equal amount of RNA was used for RT-PCR with Rho GAP specific primers. The amplification was carried out for 30 cycles (lanes 1,3,5,7) or 35 cycles (lanes 2,4,6,8). The amplified product was electrophoresed in an agarose gel.

FIGURE 5

SSCP Analysis of Rho GAP Exon



The exon 8 was preamplified with the forward and reverse primers described in Table 1 as detailed in the text. An aliquot was further amplified with radiolabeled nucleotide. The product was ron denatured and electrophoresed on a sequencing gel and autoradiographed. The lanes represent normal breast cell line MCF-10A (lanes 1 and 2), and breast carcinoma cell lines MDA-MB 231 (lane 3) and T-47D (lanes 4 and 5),

Figure 6

Nitrocellulose Overlay Assay for screening rhoGAP activity

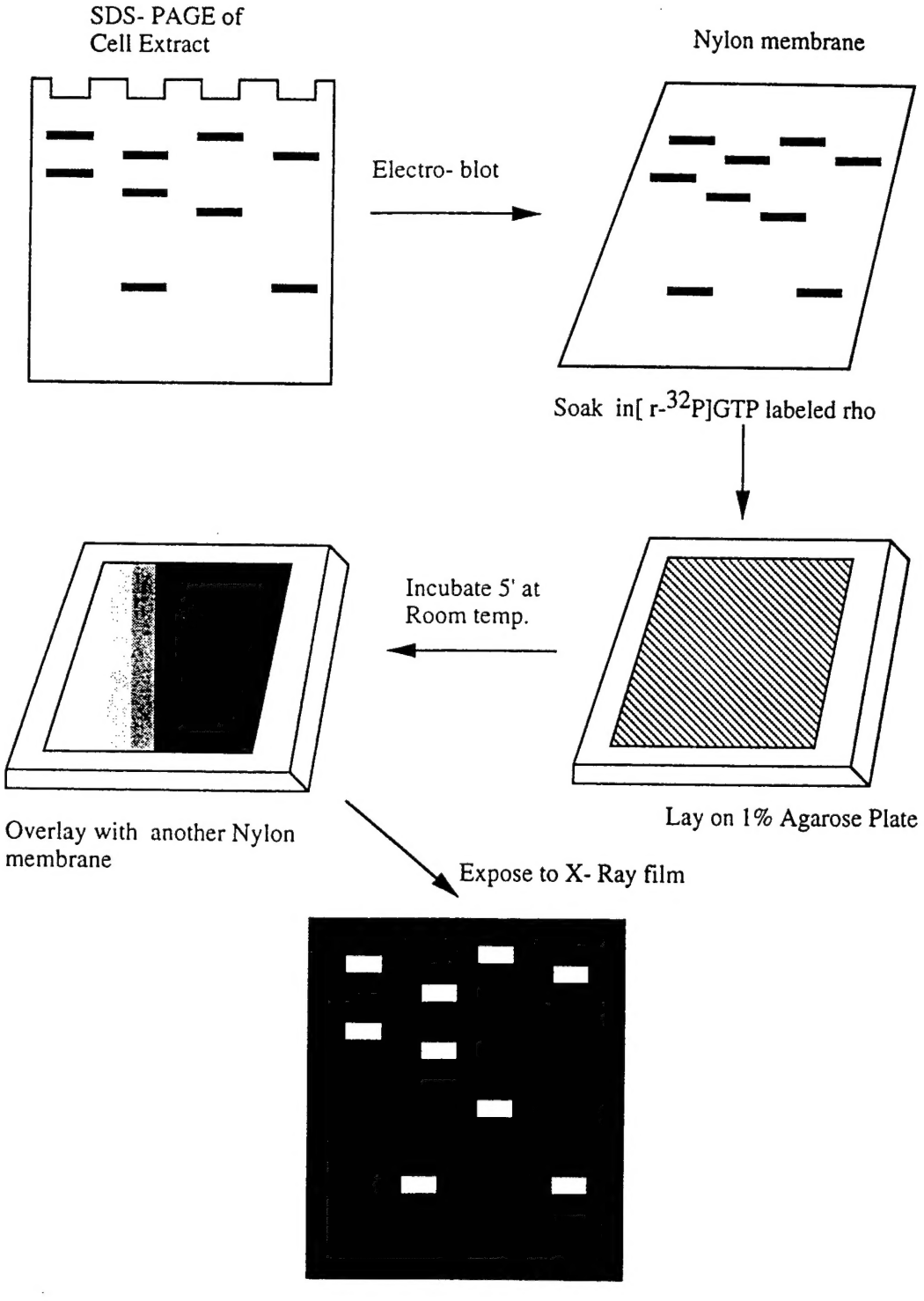


Table 1

Sequences of primers amplifying various exons of the novel RhoGAP

Primer	Sequence	Product size (~ bp.)
1F	CAA GCA CAGAGG AAA TAT TAG	
1R	AAA ATT GTC CTT AGG CTG TGC	150
2F	AAA TTG AGG CAA AAG AAG CAT G	
2R	CCT CAT ATA ACT GAG CGT ATT G	70
3F	GAT TCA CAA TTT CCC ATC AAC	
3R	CTG CAA AGA GGT TCT ACA AGG	80
4F	AGA CGA CTA AAT ACG TTG AAC	
4R	GAA GTT CAC ATC AAG TTT CAT TG	55
5aF	GGG TGA CGA CTC CGA TGA GG	
5aR	ACA TCT AGG TCC TCC AAG TAC	720
5bF	TAC TTG GAG GAC CTA GAT GTG	
5bR	CCT GTT TGG CCT GGT CAG AG	640
6F	AGG CGA CTC CGA TGG AAC AG	
6R	ACC ATG TCC AGC CGT GCT TG	180
7F	CCA AAG TTC ATG AAG AGG ATG	
7R	ACC TGA TCG AGG CAG TTG CTG	170
8F	TCA GGT GGG TCT TTT TCG CAA ATC	
8R	GAA AAT ATA AAT AAG TTT ATT TAA GC	710

Key Research Accomplishments:

*We have characterized and analyzed the sequence of a novel Rho GAP mapping to chromosome 13q12.

*The abundance of Rho GAP transcript is not detectable in some breast carcinoma cell lines, and it is generally low in breast carcinoma cell lines as compared to normal breast cell lines.

*One of the breast cancer cell lines showed a mutation in one of the exons tested.

*The abundance of Rho GAP transcript in breast carcinoma cells was responsive to growth conditions.

*Preliminary experiments indicated lower levels of Rho GAP activity in breast cancer cell lines as compared to normal breast cells.

*Coding sequences of Rho GAP, Rho A and Phospholipase C-delta were cloned in bacterial expression vector for purification of recombinant proteins for biochemical and physiological characterization of Rho GAP.

Reportable outcomes: None

Conclusions:

During the first year of the award, we have completed sequence characterization of a Rho GTPase activating protein that maps to the BRCA2 region on chromosome 13q12. Rho family of proteins shares homology with the Ras superfamily. Our working hypothesis was that breast carcinoma, where Ras mutations have not been detected, could still arise from aberrant Ras signaling by virtue of loss of activity of members of Rho family or the factors/protein that affect the activity of Rho. We have accumulated preliminary evidence to show that expression of Rho GAP, a protein which regulates the levels of active Rho, is altered in breast cancer cells. The results on the levels of Rho GAP transcript in response to growth conditions correlates to the presence of instability elements in the sequence. Thus indicating that the activity of Rho GAP in the tumor cells may be altered in one of the following three ways: a) reduced levels of transcript, b) mutations in the sequence, c) growth factors in the tumor environment. The future experiments proposed for the second and third year of the award will help further establish these observations and would also indicate the factors or proteins that interact with the Rho GAP.

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Appendices: None